A direct effect of activated human p53 on nuclear DNA replication

Citation for published version:
<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC398311/>
A direct effect of activated human p53 on nuclear DNA replication

L.S.Cox¹, T.Hupp, C.A.Midgley and D.P.Lane

CRC Cell Transformation Research Group, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK

¹Corresponding author

Communicated by D.Lane

p53 is a transcriptional activator and repressor, but recent evidence suggests that some of its many biological functions may not be dependent on transcription. To determine whether p53 exerts a direct influence on nuclear DNA replication, purified human p53 was added to a transcription-free DNA replication extract from Xenopus eggs. Full-length human p53 that inhibits SV40 DNA replication in vitro had no effect on nuclear DNA synthesis in the Xenopus system. In contrast, a C-terminal truncated form of p53 (p53Δ30), which is constitutively active for DNA binding and similar to an alternately spliced form found in vivo, showed a concentration-dependent inhibition of DNA replication in both the soluble SV40 system and eukaryotic nuclei. This inhibition occurred primarily at initiation of DNA synthesis. Oxidation of p53Δ30, which eliminates DNA binding activity, also abrogated the protein’s ability to inhibit nuclear DNA synthesis. The p53 binding DNA consensus sequence enhanced rather than competed away inhibitory activity of p53Δ30. Therefore, p53 that is constitutively active for DNA binding can inhibit nuclear DNA replication in the absence of transcription. This inhibition may require binding of p53 to DNA, in addition to interactions between p53 and proteins of the replication complex.

Key words: DNA replication/p53/Xenopus cell-free extracts

Introduction

p53 is a tumour suppressor protein that is important in maintaining genomic integrity. The protein has been postulated to act as a ‘guardian of the genome’ (Lane, 1992), monitoring the state of the cell’s DNA. p53 normally has a short half-life and is present in undetectably low amounts in normally dividing cells, but it is induced to high levels on DNA damage, resulting in arrest of cell growth and division (Kasten et al., 1991, 1992; Lu and Lane, 1993). The importance of p53 in this context can be inferred from the observation that loss or mutation of p53 is found in more than half of all human tumours (Mulligan et al., 1990; Nigro et al., 1989). The high levels of p53 induced in response to DNA damage correlate with arrest at the G1 stage of the cell cycle (Kasten et al., 1992) and this has generally been attributed to the transcriptional activity of p53 (Fields and Yang, 1990). The hypothesis of an indirect transcriptional effect of p53 on the cell cycle has been strengthened by recent reports that p53 transcriptionally induces a protein p21/WAF-1 that inhibits the activity of the cell cycle regulatory cyclin-dependent kinases in G1 (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Serrano et al., 1993; Xiong et al., 1993). Additionally, p21 has been found to bind to PCNA, the DNA polymerase δ auxiliary factor, and p21 itself can prevent synthesis of SV40 DNA in vitro, presumably via this interaction (Flores-Rozas et al., 1994; Waga et al., 1994; Warbrick et al., 1995).

However, various lines of evidence suggest that p53 can also act to suppress growth by non-transcriptional mechanisms. Some mutant p53 proteins that have lost transcriptional transactivation capacity retain their ability to arrest cell growth to varying extents (Zhang et al., 1993a,b; C.A.Midgley, unpublished results). The domains of p53 involved in transcriptional activation (N-terminal) may differ from those required for transcriptional repression (C-terminal) (Sang et al., 1994; Subler et al., 1994), suggesting that these two phenomena are separable and that growth arrest or tumour suppression may not require the transactivation capacity of p53 (Crook et al., 1994). Importantly, it has recently been demonstrated that p53-dependent apoptosis of somatotropic progenitor cells in response to X-rays occurs in the absence of new RNA or protein synthesis (Caelves et al., 1994). Conversely, loss of growth suppressor function has been reported in some p53 mutants which retain transcriptional activity (Zhang et al., 1994). However, discrepancies do exist in the literature and other authors find a very strong correlation between transcriptionally active p53 and the ability of the protein to suppress cell growth (e.g. Reed et al., 1993; Pietenpol, 1994).

It has long been known that p53 can directly prevent viral DNA synthesis by binding to and inactivating SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979; Braithwaite et al., 1987; Wang et al., 1989; Friedman et al., 1990) and p53 may compete with DNA polymerase α for binding to T antigen (Gannon and Lane, 1987). In DNA synthesis, T antigen functions as a helicase to promote strand unwinding at the replication fork, and p53 down-regulates this activity (Sturzbecher et al., 1988). Therefore, it is conceivable that p53 interacts with cellular proteins analogous to T antigen to prevent DNA replication under unfavourable conditions, e.g. when damage to the genome has been sustained. Following from this prediction, various cellular proteins have been isolated that compete with T antigen for p53 binding (Takimoto et al., 1994) and that bind to a conformation-sensitive domain of p53 (Maxwell and Roth, 1993; Iwabuchi et al., 1994). More specifically, Dutta et al. (1993) have shown that p53 interacts with the single-strand DNA binding protein RP-A and that this association is sufficient to prevent replication of viral DNA in a soluble in vitro system.
However, it has been noted that mutants of p53 that no longer arrest cell growth are still able to bind to RP-A, and p53 might even enhance DNA replication, since the transactivation domain of p53 fused to the DNA binding region of Gal4 has been found to stimulate polyomavirus or bovine papillomavirus DNA replication (He et al., 1993; Li and Botchan, 1993). On the basis of these observations, a model has been proposed where p53 binds to cellular origins of replication and there may interact with key replication proteins to block entry into S phase or to direct S phase cells into apoptosis (Pietenpol and Vogelstein, 1993). In addition, by virtue of its non-specific nucleic acid binding properties, p53 has been found to promote re-annealing of DNA and RNA strands (Oberosler et al., 1993; Brain and Jenkins, 1994), thus acting as an anti-helicase, a property that would suggest the protein should be active in preventing nuclear DNA synthesis. However, an effect on nuclear, as opposed to viral, DNA replication has not previously been experimentally addressed, possibly because of the confusion between the indirect, transcriptional contribution and a direct role.

In this paper we have therefore investigated whether p53 has a direct role in arresting nuclear DNA replication. In order to distinguish between a transcriptional role and a more direct effect of p53 in causing growth arrest, we examined the effect of purified p53 on the replication of DNA in cell-free extracts of Xenopus eggs. Such extracts support the initiation and elongation stages of DNA replication in a manner that is regulated temporally (Blow and Watson, 1987; Hutchison et al., 1987) and spatially (Hutchison and Kill, 1989; Mills et al., 1989; Cox and Laskey, 1991), but they do not support transcription (Bachvarova and Davidson, 1966).

Activity of mammalian p53 in vivo is regulated by a variety of mechanisms, including protein degradation, phosphorylation, redox, oligomerization and allosteric modification of the C-terminus (Hupp et al., 1992, 1993; Hupp and Lane, 1994). An alternately spliced form of murine p53 (p53αα) has been described in normal (Han and Kulesz-Martin, 1992) and transformed cells (Arai et al., 1986; Milner et al., 1993). This protein, which represents 25–33% of total cellular p53, is preferentially expressed in G1 of the cell cycle under normal conditions, but becomes preferentially expressed in G1 on treatment with actinomycin D (Kulesz-Martin et al., 1994), both being occasions when DNA synthesis must be suppressed. p53αα is nine amino acids shorter at the C-terminus than the major p53 and therefore would not be subject to the same allosteric modifications as the major p53. In this study, therefore, two different forms of p53 were compared for activity; full-length wild type p53 (wtP53) and a 30 amino acid C-terminal truncation (p53A30). wtP53 has latent sequence-specific DNA binding capacity that can be activated, for example by phosphorylation by casein kinase II at the C-terminus (Hupp et al., 1993; Meek et al., 1990), whereas p53A30 is constitutively active for DNA binding (Hupp et al., 1992). Correlation between the new structural models of p53 (Cho et al., 1994; Clore et al., 1994) and accumulated mutational data (Hollstein et al., 1992) suggests that DNA binding is critical for the tumour suppressor function (Friend, 1994). It is conceivable that effective mechanisms to inactivate the DNA binding or growth suppressing properties of p53 exist in the activated Xenopus egg extract in order to permit the very rapid synchronous cell cycles of alternating S and M phases of early amphibian embryos. Therefore, it was important to employ these two forms of the protein, one (wtP53) that retains the C-terminus regulatory domain and is susceptible to putative regulatory factors in the Xenopus egg extract and the other, p53A30, that should be relatively resistant to allosteric modulation. Here we show that human p53A30 inhibits nuclear DNA replication in a reversible, concentration-dependent manner, while full-length p53 with inducible DNA binding activity has no effect on nuclear DNA replication. The inhibitory activity of p53A30 is found to be ablated by oxidation of the protein. Importantly, this is the first demonstration that activated p53 can inhibit true eukaryotic nuclear, rather than viral, DNA replication in the absence of transcription.

Results

p53A30 blocks nuclear DNA replication

To determine whether p53 affected nuclear DNA replication, purified recombinant human protein, either full-length wild-type (wt) p53 or p53A30 lacking the C-terminal 30 amino acids (Figure 1), was added to Xenopus egg extract containing sperm chromatin, and DNA synthesis was analysed by incorporation of [α-32P]dATP. Figure 2A shows that replication of sperm nuclei was significantly inhibited by 20 ng/μl p53A30 and completely abolished at 40 ng/μl, whereas full-length wtP53 that had been purified under identical conditions did not appreciably affect the levels of replication of sperm nuclei. A mutant form of full-length p53 (His273) that lacks growth suppressing activity in vivo and and DNA binding capacity in vitro (Halazonetis et al., 1993) does not affect replication of sperm nuclei in this system (data not shown). Immunoblots of these samples probed with the N-terminal anti-human p53 antibody DO1 (Figure 2B) show that wtP53 and p53A30 were both equally stable during the course of the experiment. In contrast, wtP53 and p53A30 both inhibited replication of SV40 DNA in a HeLa cell extract (Figure 2C), demonstrating that the wtP53 is functionally active (Wang et al., 1989; Friedman et al., 1990). α-amanitin added to the replication extract neither decreased nuclear DNA synthesis in the presence of wtP53 nor did it relieve the inhibition imposed by p53A30 (data not shown), verifying that p53A30 blocks nuclear DNA replication in the absence of transcription.
**p53Δ30 inhibits initiation of DNA replication**

Is the replication block by p53Δ30 exerted at the level of initiation or elongation during DNA synthesis? p53Δ30 was added to egg extract at different times during replication and the amount of DNA synthesis measured at the time of p53 addition (filled columns) or after a total of 3 h incubation (hatched columns). From Figure 3A it is apparent that the inhibitory effects of p53Δ30 are greatest if the protein is added within the first 15 min of incubation. It is during this period that sperm chromatin decondenses and is assembled into intact nuclei, and that initiation of DNA replication takes place (Blow and Laskey, 1986; Blow and Watson, 1987). Immunofluorescence microscopy analysis (data not shown) revealed that nuclei were assembled normally in the presence of either wt p53 or p53Δ30 and that the proteins became localized within nuclei. Therefore, the observed inhibition was not due to a failure in nuclear assembly.

A gradual decrease in inhibitory activity was noted with increasing time of addition of p53Δ30 (Figure 3A) until, at 120 min, there was virtually no effect, probably because replication is complete and only a single round of DNA synthesis takes place in these extracts (Blow and Laskey, 1986, 1988). Since addition of p53Δ30 at times up to 60 min also resulted in some decrease in overall levels of DNA synthesis achieved, these data suggest that p53Δ30 can arrest DNA synthesis even when replication forks are actively elongating. Although we cannot rule out the possibility that initiation is not synchronous and is therefore prevented by p53Δ30 in late replicating nuclei, this explanation is less likely, since replication in this system is known to occur so rapidly that almost all replication forks must initiate synchronously (Blow and Watson, 1987; Mills et al., 1989). However, these data show that the majority of inhibition occurs at or shortly after initiation.

To further define the activity of p53Δ30, the synthesis of DNA on a single-stranded M13 DNA template was compared with that of sperm chromatin in the presence and absence of p53Δ30 (Figure 3B). While replication of sperm nuclei was strongly inhibited, the second-strand synthesis reaction on M13 was not greatly affected by p53Δ30. This aphidicolin-sensitive reaction has been likened to lagging strand DNA synthesis (Mechali and Harland, 1982) and can occur in the absence of nuclear structure (Cox and Leno, 1990). Thus p53Δ30 does not interfere directly with polymerase α/β activity in this system nor can it act simply by coating DNA, but it

---

**Fig. 2.** p53Δ30 but not full-length wt p53 inhibits nuclear DNA replication. (A) Inhibition of nuclear DNA replication by p53Δ30 (closed circles) is concentration-dependent, whereas wt p53 (open circles) does not prevent replication. (B) Immunoblot probed with anti-human-p53 monoclonal antibody DO1 following 3 h incubation of wt p53 or p53Δ30 in Xenopus egg extract (samples taken from the experiment shown in (A)), demonstrating that full-length and truncated p53 are equally stable in this system. (C) Both wt p53 (open circles) and p53Δ30 (closed circles) inhibit SV40 T antigen-dependent replication of circular plasmid DNA containing the SV40 origin of replication, as previously reported (Friedman et al., 1990; Wang et al., 1989).

---

**Fig. 3.** p53Δ30 inhibits initiation of nuclear DNA replication. (A) Time course of p53Δ30 addition to Xenopus egg extract, showing that inhibition of sperm nuclear replication is maximal when p53Δ30 is added at or before the time of nuclear envelope assembly (Blow and Laskey, 1986; Blow and Watson, 1987). Hatched columns show the final level of DNA replication after a total 3 h incubation and filled columns show the amount of replication that had taken place before p53Δ30 was added to the extract at 'time'. (B) p53Δ30 reduces replication of Xenopus sperm nuclei (sn+p53) at least 5-fold from the levels observed without added p53Δ30 (sn), but does not prevent the formation of a second strand of DNA on a single-stranded M13 DNA template (compare M13+p53 with M13 alone).
does inhibit the synthesis of DNA at replication forks within nuclei.

**Oxidation of p53Δ30 inactivates its inhibitory activity**

Since p53Δ30 prevents nuclear DNA replication and full-length wtp53 does not, it was intriguing to determine whether the difference between these two forms of p53 lay in their different abilities to bind DNA. In the first instance, therefore, the DNA binding activity of p53Δ30 was ablated. Oxidation of p53 by agents such as N-ethylmaleimide (NEM) has been found to prevent p53Δ30 from binding to DNA (Hupp et al., 1993). Therefore, we treated p53Δ30 with NEM, then quenched NEM activity using dithiothreitol (DTT) before addition to egg extract. Figure 4 shows that p53Δ30 oxidized by NEM/DTT treatment fails to inhibit replication, unlike untreated (reduced) p53Δ30. Addition of the same concentrations of NEM/DTT alone to the *Xenopus* egg extract had no significant effect on the level of DNA replication (data not shown). Incidentally, this result strongly suggests that it is p53Δ30 itself, rather than a non-specific co-purifying contaminant, that is exerting an effect on DNA synthesis. Therefore, the ability of p53Δ30 to block DNA replication in vitro is dependant on the protein existing in a reduced state and this correlates with its ability to bind DNA sequence-specifically. Hence, DNA binding by p53Δ30 is necessary for its ability to inhibit nuclear DNA replication.

**Activation of wtp53 for DNA binding does not lead to inhibition of DNA replication**

The p53Δ30 block to replication observed above may be due to the protein's enhanced double-strand DNA binding affinity compared with wtp53 (Hupp et al., 1992). Full-length human p53 can be induced to bind DNA sequence-specifically with high affinity by incubating with an anti-C-terminal monoclonal antibody, PAb421 (Hupp et al., 1992). Since DNA binding is necessary for inhibition of replication by p53, we addressed the question of whether such binding is sufficient to impose a block on DNA replication. We therefore compared replication of sperm nuclei in the presence of p53Δ30, wtp53 or wtp53 complexed with PAb421, to see if DNA binding was solely responsible for the inhibition of replication. Figure 5A clearly demonstrates that wtp53–421 behaved in the same way as wtp53 alone and did not inhibit replication of sperm nuclei in vitro, whereas p53Δ30 again showed concentration-dependent inhibition of replication. Hence, DNA binding by p53 is necessary, but not sufficient, for inhibition of nuclear DNA replication.

We further investigated whether DNA binding was the sole mechanism for the observed replication block, by competition analysis using an oligonucleotide containing the p53-specific DNA binding sequence PG (El-Deiry et al., 1992). The data in Figure 5B show that co-incubation of p53Δ30 and PG DNA did not relieve the replication block. These results suggest that this block may require p53 to bind its target DNA sequence, but that additional mechanisms, such as protein–protein interactions between p53 (bound to DNA) and replication
proteins, might enhance the inhibitory effects of p53 on DNA replication.

Discussion

This paper aimed to distinguish between transcriptional and replication roles of p53 by examining the effects of purified human p53 on nuclear DNA replication in a cell-free replication system derived from activated Xenopus eggs (Blow and Laskey, 1986) that does not support transcription (Bachvarova and Davidson, 1966). This system is exciting in that it exploits a natural developmental phenomenon that allows experimental dissociation of these two fundamental processes without resorting to damaging, artefact-inducing drugs. It is also the only cell-free system to date known to support temporally and spatially regulated replication of eukaryotic nuclei (Blow and Laskey, 1986; Blow and Watson, 1987; Hutchison et al., 1987; Mills et al., 1989; Cox and Laskey, 1991), as opposed to viral DNA synthesis in soluble systems. The early Xenopus embryo undergoes 12 synchronous and very rapidly alternating S and M phase cell cycles without intervening G phases (Laskey, 1985), so it is likely that any endogenous damage check-point controls, such as that mediated by p53, remain latent until after the mid-blastula transition (MBT), when the cell cycle lengthens and incorporates G phases (Newport and Kirschner, 1982). Xenopus laevis is tetraploid and has two genes for p53 (Soussi et al., 1987; Hoever et al., 1994). Although maternal stockpiles of p53 mRNA and protein are present in early development (Tchang et al., 1993; Cox et al., 1994; Hoever et al., 1994), it is probable that mechanisms exist in the rapidly dividing pre-MBT embryo to inactivate growth suppressor proteins such as p53, perhaps by dephosphorylation (Hupp et al., 1993). By using the truncated form of human p53, which lacks the C-terminal casein kinase II phosphorylation site (Meek et al., 1990), we have been able to overcome these putative inactivation mechanisms.

In this paper we observe that a C-terminal truncated form of p53, p53Δ30, which lacks part of the negative regulation domain and can bind DNA constitutively, inhibits nuclear DNA synthesis in a concentration-dependent manner in the Xenopus egg extract, whereas full-length wtp53 has no effect on nuclear DNA replication. The two forms of p53 were found to be equally stable during the course of these experiments, so the difference in replication effect was not due to differential degradation of wtp53 over p53Δ30. Both p53Δ30 and wtp53 inhibit synthesis of SV40 DNA in extracts of human HeLa cells. SV40 viral DNA replication in HeLa cell extract requires the activity of the essential viral replication protein, large T antigen. wtp53 is known to bind to T antigen and block its replication activities (Wang et al., 1989; Friedman et al., 1990) and we have shown that p53Δ30 equally interacts with T antigen (L.S.Cox, unpublished observations) to prevent viral DNA synthesis. The two experimental replication systems used in this paper therefore differ in that nuclear DNA replication in the Xenopus system uses normal eukaryotic synthetic machinery, while SV40 DNA synthesis depends on T antigen. This difference may account for the ability of wtp53 to inhibit SV40 but not nuclear DNA synthesis, as shown here. The concentration of p53Δ30 required to block nuclear DNA replication is suggestive of a stoichiometric requirement for p53 in replication complexes; inhibition of SV40 replication by p53 binding to and inactivating T antigen requires very similar concentrations of p53. No detectable transcription takes place in the Xenopus egg extract, suggesting that the observed replication block does not require the transcriptional activity of p53. The absence of a transcriptional component to the replication block was verified by using the RNA polymerase inhibiting drug α-amanitin.

In human cells, full-length wtp53 is thought to exert the detected cell cycle block in response to DNA damage (Kasten et al., 1991, 1992; Lu and Lane, 1993), so what is the relevance of inhibition by an artificially truncated form of the protein? An alternately spliced form of p53, which is nine amino acids shorter at the C-terminus, exists at 25–33% of the major p53 species in normal and transformed mouse cells (Han and Kulesz-Martín, 1992; Kulesz-Martin et al., 1994), thus lacking the casein kinase II phosphorylation sites and possibly also lacking other allosteric regulation sites. This protein is preferentially present at times when DNA replication does not take place, i.e. during G2 of the normal cell cycle and in G1 when the DNA has been damaged by drug treatment (Kulesz-Martin et al., 1994). It also appears probable that on DNA damage, wtp53 may change in conformation from a ‘latent’ form with weak affinity for DNA to an ‘active’ form that binds DNA more strongly (Hupp et al., 1992; Lane, 1992), possibly by C-terminal modification, such as phosphorylation (Hupp et al., 1993), alternative multimerization or even by controlled cleavage of the protein. By using p53Δ30, we have supplied an artificially activated form of p53 that cannot be inactivated by growth promoting factors in the egg extract. This form of p53 may reflect the activity of endogenous mammalian p53 in cells harbouring damaged DNA.

p53Δ30 was found to block nuclear DNA replication at an early stage, either at or soon after initiation, but it does not prevent synthesis of a second strand of DNA on a primed single-stranded M13 template. Therefore, p53 does not directly interfere with the DNA polymerases, and it appears from our data that replication forks structurally constrained within nuclei are the targets of p53 action. In favour of the hypothesis that p53 prevents initiation or early fork unwinding are the observations of Obersoser et al. (1993) and Brain and Jenkins (1994) that p53 can act to promote re-annealing of separated strands of DNA and RNA. Additionally, p53 binds to RP-A (Dutta et al., 1993) and so may promote destabilization of the single-stranded regions at the replication fork and hence enhance re-association of the strands.

DNA binding appears critical for the ability of p53 to suppress growth (Friedl, 1994), on the basis of structural (Cho et al., 1994; Clore et al., 1994) and mutational (Hollstein et al., 1991) analyses of p53. Here we show that the ability of p53Δ30 to block DNA replication correlates with its DNA binding capacity, since oxidation leads to loss of both activities. This result may be taken to imply that the inhibition of DNA replication is exerted by p53 imposing a steric block to passage of replication forks. Therefore, it is possible that by binding to DNA, either in cis (on replicating DNA) or in trans (by association with its consensus sites in other regions of the
genome), p53 may be conformationally altered in such a way as to act more efficiently as an anti-helicase or to interact more strongly with proteins of the replication complex. Interestingly, wtp53, which is usually activated for DNA binding by incubation with the C-terminal antibody PAB 421, could not be activated to block DNA synthesis, suggestive of steric effects of the bound antibody that may prevent direct associations with replication proteins.

It is probable that p53 acts on the cell cycle in multiple ways to ensure that DNA replication is prevented on genotoxic insult. First, by modulating transcription of regulatory genes such as p21/WAF-1 (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Serrano et al., 1993; Xia et al., 1993), the activity of cyclin-cdc kinase complexes is regulated and the cell cycle arrested either in G1, at the G1/S border or in S phase, via p21-mediated inhibition of cyclinE-cdc2, cyclinA-cdc2 or cyclin A-cdc2 respectively (Dulic et al., 1994). If cells manage to progress into S phase in the presence of damage, then p21 induced by p53 can bind to and inactivate the essential replication protein PCNA (Flores-Rozas et al., 1994; Waga et al., 1994; Warbrick et al., 1995). In addition to these transcriptional mechanisms, the data we present here strongly suggest that p53, when activated in vivo, can itself block DNA replication. By a combination of these three mechanisms, the cell should ensure that DNA replication cannot proceed until damage has been repaired or that the cell undergoes apoptosis rather than attempting to divide. In support of our results are the findings of Caelles et al. (1994), that show no requirement for either new RNA or protein synthesis for p53-dependent apoptosis.

In conclusion, in this paper we have managed to dissociate the putative roles of p53 in transcription and DNA replication by using a replication system in which measurable transcription does not take place and our results show that an additional mechanism for growth suppression by p53 may exist via a direct block on eukaryotic nuclear DNA replication.

Materials and methods

p53 expression and purification

Human p53 was expressed in Escherichia coli (Midgley et al., 1992) or insect Sf9 cells infected with baculovirus containing cDNA for wtp53 or p53Δ30 (Hupp et al., 1992). After biochemical purification on heparin-Sepharose and gel filtration, the proteins were microdialysed into EBB buffer (50 mM KCI, 50 mM HEPES–KOH, pH 7.4, 5 mM MgCl2, 2 mM β-mercaptoethanol, 10% glycerol, 0.1% Triton X-100). There was no appreciable difference in the activity of p53 proteins from bacterial or baculovirus sources (data not shown).

Replication reactions

Xenopus egg extract was prepared essentially as described by Blow and Laskey (1986). Replication reactions were supplemented with an energy regenerating system (150 μMcreatine phosphokinase, 60 mM phosphocreatine), 100 μM cycloheximide and 800 Ci/mmol [α-32P]dATP (Amersham). Demembranated Xenopus sperm nuclei were prepared according to the method of Gurdon (1976). Single-stranded M13 DNA was prepared by standard methods (Sambrook et al., 1989). RNase treated and purified by centrifugation on caesium chloride gradients. DNA templates were used at a final concentration of 5 ng/μl and purified wtp53 or p53Δ30 was added to various final concentrations up to 40 ng/μl. The same volume of EBB was added to negative control samples (without p53) as the volume of p53 in EBB added to the experimental samples, to control for dilution and buffer effects. Where appropriate, p53Δ30 was oxidized by treating with 5 mM NEM and then 5 mM DTT was added to quench the reaction. Reactions were carried out at 23°C for 3 h and incorporation of label determined by trichloroacetic acid precipitation followed by scintillation counting. A series of experiments using human p53 purified from baculovirus and E.coli expression systems showed inhibition of nuclear DNA replication at the same concentrations of p53 and representative examples are shown in the figures. Error bars are not shown, since Xenopus egg extracts vary from batch to batch in the absolute levels of DNA synthesis observed (see Table I in Blow and Laskey, 1986). SV40 DNA replication reactions were performed essentially as described in Wang et al. (1989), except that total reaction volumes were 10 μl. Antibody PAB421 against the C-terminus of p53 was purified on protein A-Sepharose and dialysed into EBB before mixing at equimolar concentrations with wtp53 or direct addition to the extract. PG DNA was prepared as previously described (Hupp et al., 1992), but was not radioactively labelled. Where stated, the transcriptional inhibitor α-amanitin was used at a final concentration of 5 μg/ml.

Immunoblotting

Proteins were electrophoresed on 10% SDS–PAGE, transferred to nitrocellulose and probed with undiluted tissue culture supernatant of the monoclonal antibody DO1 (Vojtisek et al., 1992). Secondary horseradish peroxidase-conjugated rabbit anti-mouse antibody was used at 1:1000 dilution and visualized by the ECL technique.

Acknowledgements

We would like to thank Dr M.Kenny for the gift of SV40 reagents. We gratefully acknowledge the Cancer Research Campaign (CRC) for its continued support and The Royal Society of Edinburgh/Caledonian Research Foundation for a personal research fellowship to L.S.C.P.D.L. is a Gibb fellow of the CRC.

References
